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PathHunter® eXpress Activated GPCR Internalization Assays

User Manual

Simple Solutions for Complex Biology

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APPENDIX A: RELATED PRODUCTS

Description	Catalog Number	For more information, visit:
Control Ligands	Many	http://www.discoverx.com/pathway_assays/control_ligands.php
AssayComplete™ Cell Plating Reagents	93-0563R0A 93-0563R5A 93-0563R27A	http://www.discoverx.com/certified cell_plating_reagents.php
PathHunter® eXpress β-Arrestin GPCR Assays	Many	www.discoverx.com/gpcrs/express_arrestin.php
PathHunter® eXpress β-Arrestin Orphan GPCR Assays	Many	www.discoverx.com/gpcrs/express_orphan.php
PathHunter® eXpress β-Arrestin Ortholog GPCR Assays	Many	www.discoverx.com/gpcrs/express_ortholog.php

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: **What is the length of compound incubation required for optimal detection of internalization and recycling of the receptor?**

A: Optimal compound incubation times are somewhat target specific. However, the majority of receptors plateau within 2 to 3 hours. Therefore, the PathHunter eXpress Activated GPCR Internalization assays were developed using a single, universal protocol that includes a 3 hour compound incubation step.

Q: Is the EFC signal impacted by a change in pH?

A: No. The enzyme fragments are always in the cytosol, so the pH does not change.

Q: How much internalization is required before signal is detected?

A: The amount of internalization that occurs prior to ligand stimulation will definitely vary from target to target. Activated Internalization assays are highly specific such that even if the receptor is internalizing (in the absence of ligand) you don't get signal because Arrestin is not recruited and EA is not present at the endosome with the receptor, thus no complementation occurs.

Q: Can my eXpress assay be run in 384-well format?

A: All PathHunter eXpress assays are optimized and formatted to run in 96-well plates. Certain assays also perform well in 384-well format but require modifications to the protocol (cell numbers, incubation times, etc.). Please contact Technical Support (techsupport@discoverx.com) for more information.

For additional information or technical support, please call 1.866.448.4864 (NA) or +44.121.260.6142 (Europe) or email us at info@discoverx.com.

LEGAL SECTION

This product and/or its use is covered by one or more U.S. patents #7,135,325 B2, #8,101,373 B2, and/or foreign patent applications and trade secrets that are either owned by or licensed to DiscoveRx® Corporation.

LIMITED USE LICENSE AGREEMENT

The designated cells and reagents purchased from DiscoveRx are restricted in their use. DiscoveRx has developed an assay for translocation and internalization("Assay") employing genetically modified cells ("Cells") and detection reagents ("Reagents") (collectively referred to as "Materials"). The Cells and Reagents are designed and optimized to be used together in the Assay. DiscoveRx wishes to ensure that these Cells and Reagents are used properly and effectively. By purchasing the Materials you recognize and agree to the restrictions.

1. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by DiscoveRx.
2. Purchaser will not analyze the Reagents nor have them analyzed on Purchaser's behalf.
3. Purchaser will use only the Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Assays.

If the purchaser is not willing to accept the limitations of this limited use statement and/or has any further questions regarding the rights conferred with purchase of the Materials, please contact:

Licensing Department
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Fremont, CA 94538 USA
tel | 1.510.979.1415 x104
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For some products/cell lines, certain 3rd party gene specific patents may be required to use the cell line. It is the purchaser's responsibility to determine if such patents or other intellectual property rights are required.

INTENDED USE

PathHunter® eXpress Activated GPCR Internalization Assays are ready to use complete kits that contain everything you need to measure arrestin bound internalized GPCRs in live cells without the hassle of using antibodies, radioactivity or elaborate imaging. The eXpress kits include single use vials of frozen cells stably expressing the GPCR of interest, optimized cell plating reagent, chemiluminescent detection reagents and plates*. Simply thaw and plate the pre-validated cells and challenge with compound 24 or 48 hours later. Whether you are studying receptor recycling, identifying functional antagonists, or determining mechanism of action of your lead compounds, the ready-to-assay eXpress format eliminates the need for lengthy, expensive and time consuming cell culture and makes functional testing fast and convenient. Assays are designed for 96-well plate analyses and kits include enough cells and detection reagents for either 100, 200 or 1000 datapoints.

*Test compounds are not included and must be provided by the researcher.

TECHNOLOGY PRINCIPLE

PathHunter eXpress Activated GPCR Internalization Assays provide a quantitative measurement of internalized GPCR protein localized to early endosome using β-galactosidase (β-gal) enzyme fragment complementation (EFC, Figure 1). In this system, the small, 42 amino acid enzyme fragment of β-gal called ProLink™ (PK) is localized to intracellular endosomes and the larger, complementing enzyme fragment termed Enzyme Acceptor, or EA, is fused to β-Arrestin. Stimulation of the receptor results in Arrestin binding to the activated GPCR, followed by internalization and trafficking of the receptor/arrestin complex to cellular endosomes. This action forces complementation of the two enzyme fragments, resulting in the formation of a functional enzyme that is capable of hydrolyzing substrate and generating a chemiluminescent signal using PathHunter Detection Reagents.

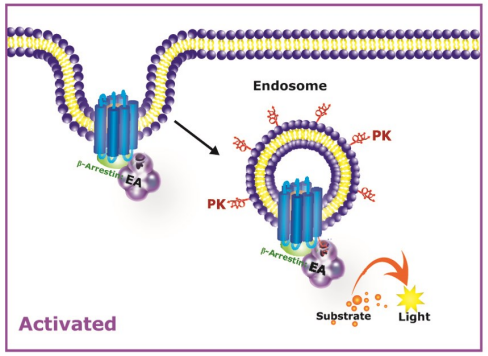


Figure 1. PathHunter® eXpress Activated GPCR Internalization Assay Principle. Activation of the GPCR results in internalization of the receptor to intracellular endosomes and formation of a functional β-gal enzyme capable of hydrolyzing substrate and generating chemiluminescent signal.

FREQUENTLY ASKED QUESTIONS (CONTINUED)

Q: Why do longer incubation times with Detection Reagents lead to a higher signal?

A: The complemented β-galactosidase (β-gal) enzyme is continually turning over the substrate over time. Theoretically, the signal continues to increase until the substrate is depleted. Therefore, the longer you incubate the reaction, the higher the RLU values.

Q: What is the shelf life of the eXpress kits?

A: We recommend that eXpress kits should be used within 6 months of receipt under proper storage conditions. For short term (2 weeks or less), store eXpress cells at −80°C. For long term storage (more than 2 weeks), store in the vapor phase of liquid nitrogen (N_2). Store the Detection Reagent Kit at −20°C. Refer to the kit label for lot specific expiration date information.

Q: What if my Cell Plating Reagent changes from a red/pink color to yellow after freezing/thawing?

A: If the Cell Plating Reagent changes color from red/pink to yellow after thawing, please continue with the assay according to the product insert. We have observed this color change on rare occasions and have confirmed that it will not affect assay performance.

Q: Can I use this assay to test human plasma or serum samples?

A: Yes. PathHunter eXpress Activated GPCR Internalization assays tolerate up to 80% serum or plasma. First, prepare a standard curve of spiked ligand in neat, heparinized plasma (or mouse, human serum). Add samples directly to the cells (no further dilution – 100% plasma in the well). After stimulation, remove the plasma or serum sample and replace with fresh CELL PLATING reagent before addition of the PathHunter Detection Reagents. It has been shown that EDTA anti-coagulated plasma inhibits EFC and should be avoided for these types of studies.

Q: What instruments can I use to read the plates?

A: Any bench top luminometer will work with the PathHunter eXpress Activated GPCR Internalization Assays. Below is a partial list of commercially available luminometers that have been used to validate our assays:

Turner Biosystems: Modulus Microplate
GE Healthcare Life Sciences: LEADseeker™, FarCyte™
BMG Labtech: PHERAstar Plus, LUMIstar Omega
Perkin Elmer: TopCount®, VICTOR II or V, Fusion, LumiCount, EnVision, MicroBeta® (Trilux), ViewLux
Molecular Devices: CLIPR™, LJL Acquest, LJL Analyst, LJL Analyst HT, LJL Analyst GT, Gemini, SpectraMax®, Flexstation™, LMax
Tecan: Ultra Evolution
Beckman Coulter – CRi
Berthold Technologies: Mithras LB 940
Hamamatsu: FDSS6000, FDSS/RayCatcher

FREQUENTLY ASKED QUESTIONS

Q: I did not see a signal with my control agonist.
A: There may be differences in agonist purchased from different vendors. Confirm that the control agonist used is the same ligand used in the dose response shown in the provided cell-line specific data sheet.

Q: Can the source of my agonist or antagonist compound impact my assay performance?
A: Yes, the vendor/source of compound can impact assay performance dramatically. Compounds can vary in purity from vendor to vendor. In addition, vendors will recommend different diluents (methanol, NaOH, ethanol, DMSO, water), different treatments (boiling, freeze/thaw, etc) or different storage temperatures for the same compound. Each PathHunter eXpress target has been QC tested and validated using a reference ligand. Information on the reference ligand used for each assay (including the vendor source and catalog number) can be found on the cell line specific datasheet. For optimal assay performance, we recommend using control ligands provided by DiscoveRx. Visit www.discoverx.com/ligands/control_ligands for the complete DiscoveRx offering.

Q: I did not see a response with my compound.
A1: The concentration of DMSO or Ethanol used for dilution is too high. Maintain concentration of the agonist/antagonist diluent at ≤ 1%.
A2: Confirm that the final ligand concentration is correct. Some ligands are "sticky" and difficult to dissolve.
A3: Confirm that the cell line responds to the control agonist.

Q: My cells arrived thawed. Can I use them?
A: No. Call technical support for a replacement.

Q: How long is the prepared detection reagent good for?
A: The working detection reagent solution must be used within 8 hours of mixing.

Q: How long is the signal stable for?
A: The signal is stable for 24 hours after addition of detection reagent.

Q: My cells are floating after the 48 hours incubation.
A: The cells are not viable, contact technical support for a replacement.

Q: Can I switch plates or should I use the plate provided?
A: You can use any clear bottom white or opaque walled plate.

Q: What if cells are not completely adherent after 24/48 hrs incubation?
A: For certain targets, cells may not be completely adherent after 24 hours, but still greater than 80% viable. Please continue on with the protocol as described in the user manual.

PROTOCOL OVERVIEW

Please read the entire protocol completely before running the assay. Successful results depend on performing these steps correctly. Refer to the cell-line specific datasheet for additional information on optimized cell plating reagent and reference ligand. For additional information or Technical Support, contact DiscoveRx or visit www.discoverx.com.

The following steps are required to monitor the fate of activated and internalized GPCRs using a PathHunter eXpress Activated GPCR Internalization assay (Figure 2).

1. Thaw and plate frozen, assay-ready eXpress cells (page 9).
2. Dilute and add compounds.
3. Perform functional assay in agonist (page 10) or antagonist mode (page 13).

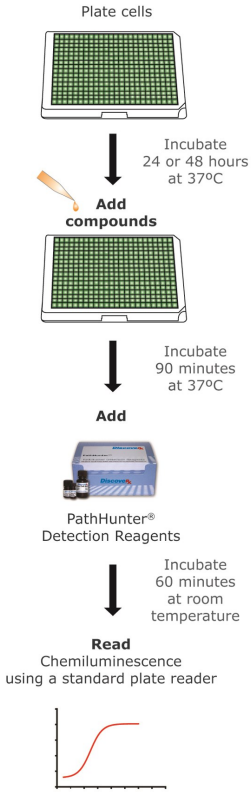


Figure 2. Monitor functional GPCR responses to compound challenge using the fast and simple PathHunter eXpress Activated GPCR Internalization assay protocol.

KIT CONTENTS AND STORAGE CONDITIONS

PATHHUNTER EXPRESS ACTIVATED GPCR INTERNALIZATION KIT COMPONENTS REQUIRE MULTIPLE STORAGE TEMPERATURES. OPEN BOXES IMMEDIATELY AND STORE CONTENTS AS INSTRUCTED.

SHELF LIFE:
Use kit within 6 months from the date of receipt under proper storage conditions.

BOX 1: PATHHUNTER EXPRESS ACTIVATED GPCR INTERNALIZATION CELLS:

STORAGE:
Short term (2 weeks or less): **Store vials -80°C immediately upon arrival.**
Long term (greater than 2 weeks): **Place vials in the vapor phase of liquid nitrogen (N_2).**

PathHunter eXpress Activated GPCR Internalization cells arrive frozen on dry ice. Cells are delivered in individual vials containing $1x10^6$ cells in 100 µL of freezing medium. Each vial contains sufficient cell numbers to generate (1) 96-well microplate prepared at the seeding density described.

When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid N_2 inside the vial to evaporate and proceed with the thawing protocol (page 9). **Do not touch the bottom of the tubes at any time to avoid inadvertent thawing of the cells. If cells are not frozen upon arrival, do not proceed. Contact technical support.**

BOX 2: PATHHUNTER DETECTION REAGENT AND CP REAGENT: Store at -20°C

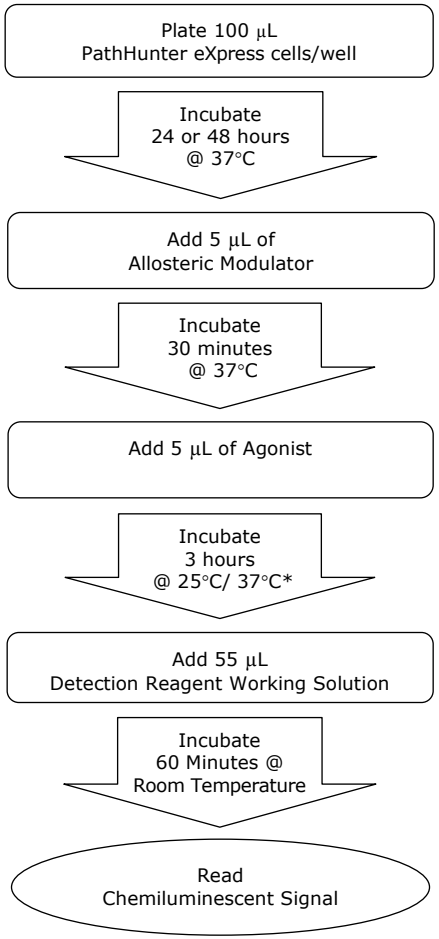
Once thawed, store the Cell Plating (CP) Reagent at 4°C. Avoid multiple freeze/ thaw cycles. In rare instances, the CP Reagent may be yellow in color after thawing. Although this indicates a slight change in pH, continue with the assay as this does not impact assay performance.

Thaw the PathHunter Detection Reagents at room temperature before use, and after thawing, store reagents for up to 7 days at 4°C. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance. Once made, the working solution is stable for 24 hours at room temperature.

BOX 3: 96-WELL TISSUE CULTURE TREATED PLATES: Store at Room Temperature

3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your allosteric modulator dose response.

QUICK-START PROCEDURE: ALLOSTERIC MODULATOR DOSE RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

e) Remove 30 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f) With a clean pipet tip, remove 30 µL of diluted compound from tube #11, add it to the tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g) Repeat this process 7 more times, preparing serial dilutions from right to left across the plate. **DO NOT add modulator compound to tubes #1 and 2.** These samples serve as the no modulator control and complete the dose curve.
h) Repeat process when testing additional compounds.
i) Set compounds aside until they are ready to be added.

3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes #1-12 to each well according to the plate map on page 17.
5. Incubate for 30 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the modulator compound incubation, determine the EC_{10} and EC_{90} concentration of the agonist from the agonist dose response curve (described on pages 10 - 12). Prepare a **22X** EC_{10} concentration (PAM) or **22X** EC_{90} concentration (NAM) of agonist compound as shown below:

   Example: If the EC_{10}/EC_{90} of the agonist compound is 10 nM, prepare a stock at 220 nM.
2. Add 5 µL of agonist compound to each well. Add 5 µL of CP reagent to the no agonist wells (column 1).
3. Incubate for 3 hours @ 25°C/37°C*****.

NOTE:
*****Please refer to the cell line specific datasheet for any variation in assay conditions.

SUBSTRATE PREPARATION AND ADDITION

1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**

MATERIALS PROVIDED

Description	Contents			Storage
Box 1: PathHunter eXpress Activated GPCR Internalization Cells	1 vial $1x10^6$ cells ea	2 vials $1x10^6$ cells ea	10 vials $1x10^6$ cells ea	-80°C (short) Liquid N_2 (long)
Box 2: PathHunter Detection Reagents - Cell Assay Buffer - Substrate Reagent 1 - Substrate Reagent 2***** Cell Plating Reagent‡	100dp 5.7 mL 1.5 mL 0.3 mL 1 X 20.0 mL	200 dp 9.5 mL 2.5 mL 0.5 mL 2 X 20.0 mL	1000 dp 57.0 mL 15.0 mL 3.0 mL 2 X 100 mL	-20°C
Box 3: 96-well Tissue Culture Treated Plates	1 plate	2 plates	10 plates	Room Temp

‡Refer to cell-line specific data sheets for optimized Cell Plating Reagent included with each kit.
*Centrifuge vial before opening to maximize recovery.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

The following additional materials are required but not provided:

1. Pipettes and pipette tips
2. Tissue culture disposables
3. GPCR control agonist as recommended in the cell line specific datasheet. Visit www.discoverx.com/pathway_assays/control_ligands.php for the complete DiscoveRx offering
4. GPCR test compound(s)
5. Disposable Reagent Reservoir such as Thermo Scientific, Cat. #8094 or similar
6. 96-well V-bottom compound dilution plates (DiscoveRx, Cat. #92-0011)
7. Multi-mode or luminescence plate reader.

RECOMMENDED MATERIALS

The following products* are recommended:

- CytoTracker™ LDH Quantification Kit (DiscoveRx, Cat. # 92-2002)
- CytoTracker™ Glutathione Quantification Kit (DiscoveRx, Cat. # 92-2003)
- CytoTracker™ DNA Damage Quantification Kit (DiscoveRx, Cat. # 92-2004M)

* Products not available in all countries. Please inquire.

ASSAY INCUBATION AND CELL PLATING REAGENT REQUIREMENTS

Each PathHunter eXpress Activated GPCR Internalization assay has been validated for optimal assay performance at either 24 or 48 hours post-thaw. Although most targets perform similarly at both time points, for optimal assay performance we recommend you perform the assay according to the protocol provided in the cell line specific datasheet using both the recommended time point and CP Reagent. **Always use the CP Reagent included in the kit and DO NOT substitute from an alternate kit at any time.**

NOTE:
Use special caution when testing multiple targets in the same experiment as targets may have different incubation times and CP Reagent requirements.

COMPOUND PREPARATION AND INCUBATION TEMPERATURE

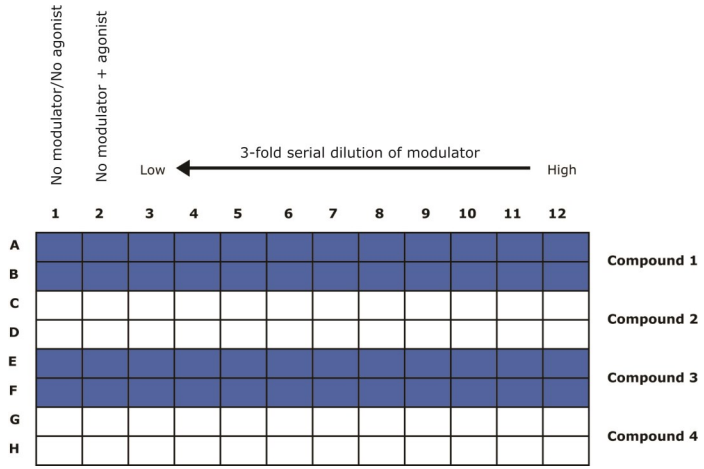
PathHunter eXpress Activated GPCR Internalization Assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). As solvents can affect assay performance, optimize the assay conditions accordingly if other solvents or solvent concentrations are required.

To validate each PathHunter eXpress Activated GPCR Internalization Assay, reference ligand was diluted in the recommended CP Reagent containing appropriate solvent. For preparation of test compounds, we recommend preparing the dilutions using the CP Reagent provided in the kit. For antibodies or other compounds that may be sensitive to serum and/or other assay components, dilutions can be prepared in either Hanks Buffered Salt Solution (HBSS) + 10 mM HEPES + 0.1% Bovine Serum Albumin (BSA) or OptiMEM® + 0.1% BSA without affecting assay performance.

The kinetics of ligand-induced receptor internalization can vary depending on the target and temperature used during the compound incubation step. For optimal assay performance, we recommend you perform the compound incubation step according to the protocol provided in the cell line specific datasheet. **Always use the incubation temperature recommended for the kit you are testing.**

ASSAY PROCEDURE — ALLOSTERIC MODULATOR DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing allosteric modulator assays using the PathHunter eXpress Activated GPCR Internalization cells and PathHunter Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing an 11-point dose curve for each compound concentration using at least *duplicate* wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: MODULATOR COMPOUND PREPARATION AND ADDITION

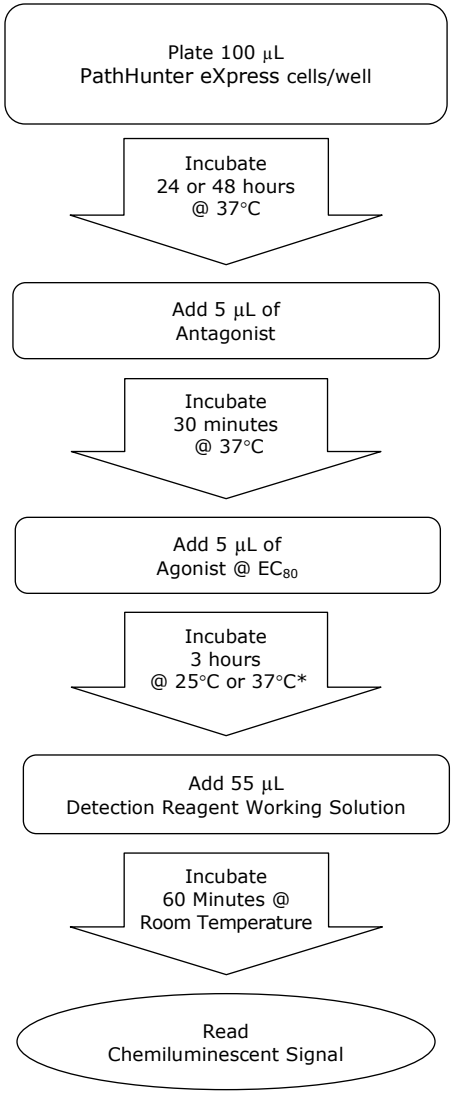
1. Dissolve modulator compound in the vehicle of choice (DMSO, Ethanol, PBS or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of modulator compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL modulator compound will be used in a final colume of 110 µL). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 11-point dose curve serial dilutions: We recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (**1100X** IC_{50} would be the final working concentration).

   Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration at 11 µM. This is the working concentration.

   a) Label tubes 1 through 12.
   b) Add 60 µL of CP reagent containing appropriate solvent to tubes #1-11.
   c) Prepare a working stock of modulator compound in the appropriate CP reagent.
   d) Add 90 µl of the working concentration of modulator compound to tube #12.

QUICK-START PROCEDURE: ANTAGONIST DOSE RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

USE OF PLASMA OR SERUM CONTAINING SAMPLES

PathHunter eXpress Activated GPCR Internalization Assay can be run in the presence of high levels of serum or plasma without negatively impacting assay performance. Standard curves of control ligand can be prepared in neat, heparinized plasma and added directly to the cells (without further dilution, ie. 100% plasma in the well). After ligand stimulation, the samples should be removed and replaced with fresh CP Reagent before the addition of the PathHunter Detection Reagents.

NOTE:
EDTA anti-coagulated plasma samples do not give a positive response in the assay. Therefore, the choice of anti-coagulant treatment is very important.

THAWING AND PLATING FROZEN CELLS

The following steps outline the procedure for thawing and plating frozen PathHunter eXpress Activated GPCR Internalization cells from freezer vials:

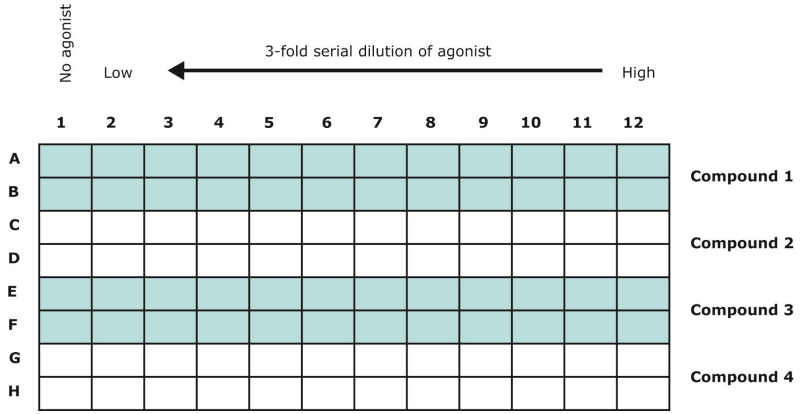
1. Pre-warm CP Reagent in a 37°C water bath.
2. Remove cell vial(s) from -80°C or liquid N_2 vapor phase storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**

   NOTE:
   When removing cryovials from liquid N_2, place immediately on dry ice in a covered container. Wait at least one minute before opening for any liquid N_2 inside the vial to evaporate.
3. Place the cell vial(s) ***briefly*** (10 seconds to 1 min) in a 37°C water bath until only small ice crystals remain and the cell pellet(s) is almost completely thawed.
4. Add 0.5 mL of pre-warmed CP Reagent to the cell vial. Pipette up and down gently several times to ensure that the cells are evenly distributed.
5. Immediately transfer the cells to 11.5 mL of pre-warmed CP Reagent and pour into a disposable reagent reservoir.
6. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.
7. After seeding the cells into the microplate, incubate for either 24 or 48 hours at 37°C, 5% CO_2.

   NOTE:
   *Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE - AGONIST DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing agonist assays using the PathHunter eXpress Activated GPCR Internalization cells and PathHunter Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing a 12-point dose curve for each compound using at least duplicate wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: AGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve agonist compound in the vehicle of choice (DMSO, Ethanol, PBS or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of agonist compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **11X** of the final screening concentration (i.e. 10 µL compound + 100 µL of cells). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 12-point dose curve serial dilutions:
   We recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (**550X** EC_{50} would be the final screening concentration).

   Example: If the expected EC_{50} is 10 nM, prepare the highest starting concentration at 5.5 µM.

   a. For each compound tested, label tubes 1 through 12.
   b. Add 60 µL of CP Reagent to tubes #1-11.
   c. Prepare a working concentration of agonist compound in appropriate CP Reagent.
   d. Add 90 µL of the working concentration of agonist compound to tube #12.
   e. Remove 30 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.

SUBSTRATE PREPARATION AND ADDITION

1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your antagonist dose response.

a. Label tubes 1 through 12.
b. Add 60 µL of CP Reagent to tubes #1-11.
c. Prepare a working stock of antagonist compound in the appropriate CP Reagent.
d. Add 90 µL of the working concentration of antagonist compound to tube #12.
e. Remove 30 µL of diluted compound from tube #12, add it to tube #11 and mix gently by pipetting up and down. Discard the pipet tip.
f. With a clean pipet tip, remove 30 µL of diluted compound from tube #11, add it to the tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g. Repeat this process 7 more times, preparing serial dilutions from right to left across the plate.
h. **DO NOT add antagonist compound to tubes #1 and 2**. These samples serve as the no antagonist controls and complete the dose curve.
i. Repeat process when testing additional compounds.
j. Set compounds aside until antagonist compounds are ready to be added.

3. Remove PathHunter eXpress Activated GPCR Internalization cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes 1-12 to each well according to the plate map on page 13.
5. Incubate for 30 minutes at 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the antagonist incubation, determine the EC_{80} concentration of the agonist from the agonist dose response curve (described on pages 10-12). Prepare a **22X** EC_{80} concentration of agonist compound as shown below:

   Example: If the EC_{80} of the agonist compound is 10 nM, prepare a stock at 220 nM.
2. Add 5 µL of agonist compound to each well. Add 5 µL of CP Reagent to the no agonist wells (column 1).
3. Incubate for 3 hours @ 25°C or 37°C*.

   ***NOTE:**
   Please refer to the cell line specific datasheet for any variation in assay conditions.

f. With a clean pipet tip, remove 30 µL of diluted compound from tube #11, add it to tube #10 and mix gently by pipetting up and down. Discard the pipet tip.
g. Repeat this process 8 more times, preparing serial dilutions from right to left across the tubes.
h. **DO NOT add agonist compound to tube #1**. This sample serves as the no agonist control and completes the dose curve.
i. Repeat this process for each compound to be tested.
j. Set compounds aside until agonist compounds are ready to be added.

3. Remove PathHunter eXpress Activated GPCR Internalization cells (previously plated on day 1) from the incubator.
4. Transfer 10 µL from tubes #1-12 to each well according to the plate map on page 10.
5. Incubate for 3 hours @ 25°C or 37°C*.

   ***NOTE:**
   Please refer to the cell line specific datasheet for any variation in assay conditions.

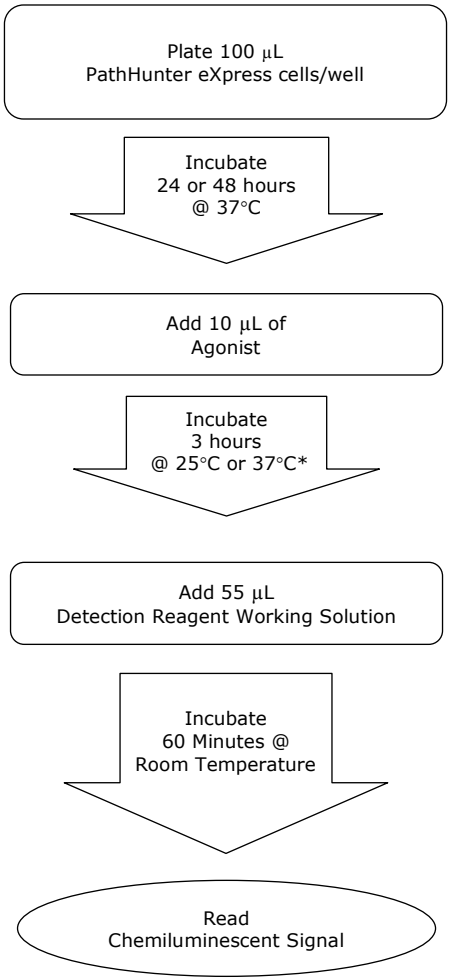
SUBSTRATE PREPARATION AND ADDITION

1. During the incubation period, prepare a working stock of PathHunter Detection Reagents by mixing **19 parts** Cell Assay Buffer, **5 parts** Substrate Reagent 1 and **1 part** Substrate Reagent 2.

Component	Entire Plate (96 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

   NOTE:
   The working solution is stable for up to 8 hours at room temperature.
2. Add 55 µL of prepared detection reagent per well and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
3. Read samples on any standard luminescence plate reader.
4. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

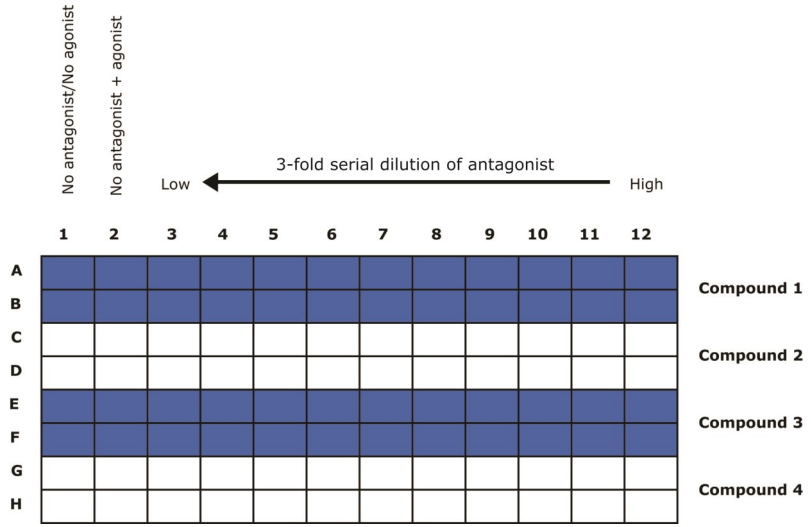
QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE — ANTAGONIST DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing antagonist assays using the PathHunter eXpress Activated GPCR Internalization cells and PathHunter Detection Reagents. Although plate layouts and experimental designs may vary, we recommend performing an 11-point dose curve for each compound using at least *duplicate* wells for each dilution. The protocol and volumes described below are designed for a complete 96-well plate.



DAY 2 OR 3: ANTAGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve antagonist compound in the vehicle of choice (DMSO, Ethanol, PBS or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of antagonist compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL antagonist compound will be used in a final volume of 110 µL). For each dilution, the final concentration of solvent should remain constant.

   Preparation of 11-point dose curve serial dilutions:
   We recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (**1100X** IC_{50} would be the final screening concentration).

   Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration at 11 µM.